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The beneficial effects of treatment with tamoxifen and anti-oestradiol antibody on experimental systemic lupus erythematosus are associated with cytokine modulations

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SUMMARY

In an attempt to elucidate the role of oestrogens in systemic lupus erythematosus (SLE) we investigated the effects of treatment with an oestrogen antagonist – tamoxifen and a monoclonal anti-oestradiol (anti-E2) antibody on mice in which experimental systemic lupus erythematosus (SLE) was induced by a human monoclonal anti-DNA antibody bearing the 16/6 idiotype (16/6 Id). Thus, groups of BALB/c female mice were immunized with the 16/6 Id and 3 weeks following the booster injection, when antibody titres were elevated in the injected mice, treatment protocols with anti-oestradiol or tamoxifen were initiated. Control groups that were not immunized with the 16/6 Id but were similarly treated with the above agents were included in the study. The treatment with the above agents had no effect on the total autoantibody titres; however, a decrease in the immunoglobulin G (IgG)2a/IgG_1 ratio of the anti-DNA antibodies was determined in the 16/6 Id immunized and treated mice. Further, both the anti-oestradiol and tamoxifen had beneficial effects on the clinical manifestations (white blood cell counts, levels of protein in the urine and immune complex deposits in the kidneys) of the 16/6 Id immunized and treated mice. We have previously observed a significant elevation in interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) secretion in mice with experimental SLE and a reduction in IL-2, IL-4 and interferon-γ (INF-γ) levels as compared with the levels detected in healthy controls. Treatment with either the anti-oestradiol antibody or with tamoxifen restored the levels of all the above cytokines to the normal levels observed in the control mice.These findings suggest that cytokine modulation may be the basis for the therapeutic effects of both anti-oestrogens in experimental SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a classic autoimmune disease that is characterized by the production of autoantibodies against self antigens such as DNA, histones, ribonucleoprotein, Sm, Ro (SS-A), and La (SS-B).[1] We have reported the induction of an experimental SLE-like disease in naive mice of different strains that do not develop autoimmune disorders spontaneously.[2] The induction of experimental SLE was first achieved by immunizing mice with a human anti-DNA monoclonal antibody that expresses a common idiotype designated 16/6 Id.[3] Following immunization with the 16/6 antibody, high levels of anti-16/6 Id, anti-anti-16/6 Id, anti-DNA, and antibodies to nuclear antigens were detected in the immunized mice. The serological findings were associated with increased erythrocyte sedimentation rates, leucopenia, proteinuria, abundance of immune complexes in the kidneys, and sclerosis of the glomeruli.[2] Some mice developed other clinical manifestations such as alopecia or paraparesis/paraplegia. Thus experimental SLE resembles in many aspects SLE in man.

A differential sex susceptibility to various autoimmune diseases has long been recognized in man. Thus, in general, women have a greater preponderance of these diseases.[4–6] In SLE, for example, the female to male susceptibility ratio is 9:1.[4] A similar sex related expression of disease is known to occur in several animal models of autoimmune diseases.[4,7,8] While oestrogens were shown to enhance the disease process an amelioration of the autoimmune disease following androgen administration was seen.[9] We have also demonstrated that orchiectomized BALB/c male mice that were treated with oestrogen prior to their immunization with the 16/6 Id developed a very active disease with an early onset as compared to the mild disease observed in BALB/c males that were only immunized with the 16/6 Id.[10] Attempts to treat BALB/c female mice in which experimental SLE has been induced by

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Abbreviations: ACTH, adrenocorticotropic hormone; NE, nuclear extract; WBC, white blood cells.

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the 16/6 Id, with the oestrogen antagonist – tamoxifen – demonstrated beneficial effects on the SLE-related clinical manifestations.[11]

The mechanism of action of sex hormones on the immune system is unclear and it may be either a direct one via steroid receptors on target organs or indirect via some factors such as cytokines. Reports have indicated that various cytokines might be responsive to sex hormones.[12–16] It is possible that many of the effects seen in SLE might be due to the excess or absence of cytokines. We have previously shown a cytokine dysregulation in mice afflicted with experimental SLE induced by immunization with the 16/6 Id.[17] Thus, an endocrine–immune regulatory network might exist which affects the balance between the T helper 1 (Th1)- and Th2-type responses and profile of the resulting cytokines.[18]

In a previous study we observed that tamoxifen had beneficial effects on the manifestations of experimental SLE in female BALB/c mice.[11] The present study was aimed at determining whether the effect of tamoxifen is due to its being an oestrogen antagonist. In addition, we wanted to find out whether cytokines play a role in the modulation of the experimental disease. We report here that the effect of tamoxifen on the disease manifestations appears to be via its properties as an oestrogen antagonist since a monoclonal anti-oestradiol (anti-E2) antibody had the same beneficial effects on the clinical manifestations of the disease. Moreover, treatment with either tamoxifen or anti-E2 reversed the abnormal secretion of cytokine observed in mice afflicted with experimental SLE to a profile shown in healthy controls.

MATERIALS AND METHODS

Mice

BALB/c female mice were purchased from Olac (Bicester, Oxfordshire, UK). Mice were used at the age of 6–8 weeks.

Antibodies

The human monoclonal antibody (mAb) 16/6 is an anti-DNA antibody that expresses a common idiotype, 16/6 Id.[19] The hybridoma that secretes the antibody was grown in culture and the antibody was isolated from the culture supernatant by affinity chromatography on a protein G column (Pharmacia, Uppsala, Sweden). For coating plates for enzyme-linked immunosorbent assay (ELISA), mAb 16/6 Id was used at a concentration of 10 μg/ml.

The monoclonal anti-oestradiol antibody (monoclonal anti-oestrogen, anti-E2) was originated from a fusion of mouse myeloma cells P3/NSI/1 with the spleen cells of a C57BL/6 female mouse immunized with oestradiol-6-carboxymethyl bovine serum albumin (BSA).[20] The clone used (clone 15) had high affinity ($K_a = 10^{-9}$ M) to oestradiol and was of the immunoglobulin G (IgG)2b isotype. The hybridoma was grown as ascites in the peritoneum of (C57BL/6 × BALB/c)F1 male mice and the antibody was purified from the ascitic fluid using a Protein-A–Sepharose (Pharmacia) column.

The rabbit anti-16/6 Id was produced by the immunization of rabbits with the human 16/6Id.[21] The antibodies were affinity purified from the rabbit sera on a goat anti-rabbit immunoglobulin–Sepharose column and the eluate containing anti-16/6 Id antibodies was used for coating of ELISA plates at a concentration of 5 μg/ml.

Antigens

DNA. For identification of anti-DNA antibodies, plates were coated with 50 μl/well of 10 μg/ml methylated BSA (Sigma, St. Louis, MO). The plates were then washed and coated with 50 μl of 10 μg/ml of either denatured (boiled for 15 min and cooled rapidly on ice) calf thymus DNA (Sigma) or native calf thymus DNA.

HeLa nuclear extract (NE). NE was prepared as described previously[22] and stored at −70°. NE was used for coating ELISA plates (50 μl/well) at a concentration of 5 μg/ml.

Immunization and treatment of mice

Groups of BALB/c female mice (10–15 mice per group) were injected with the human monoclonal 16/6Id (1 μg/mouse) in complete Freund's adjuvant (CFA) (Difco, Detroit, MI) intradermally in the hind footpads and were boosted with the same amount of antibody in phosphate-buffered saline (PBS) 3 weeks later. A non-immunized group of mice served as control. Three weeks following the booster injection the mice were bled and their sera were tested for production of antibodies of various specificities. Upon confirmation of presence of DNA and 16/6 Id specific antibodies in the sera of immunized mice they were divided into groups and tamoxifen and anti-oestrogen (anti-E2) treatment commenced. Tamoxifen was injected into mice (800 μg/mouse) twice a week (subcutaneously into the neck). The IgG purified fraction of the monoclonal anti-E2 antibody was injected into the appropriate experimental group weekly (50 μg/mouse) intraperitoneally. Control, non-immunized mice, were treated with either tamoxifen or anti-E2 using the same treatment protocols.

ELISA

Maxisorb microtitre plates (Nunc, Roskilde, Denmark) were coated with one of the above antigens or antibodies in the above stated concentration. Thereafter the plates were blocked with 1% ovalbumin (Sigma), in PBS, and the sera of the mice, diluted serially from 1:10–1:1280 were incubated for 90 min. Plates were then washed and incubated for 60 min with goat anti-mouse IgG (γ chain specific) conjugated to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). For the determination of the immunoglobulin isotypes of the antibodies assayed horseradish peroxidase-labelled goat anti-mouse IgG_1 (γ_1 chain specific) or IgG_{2a} (γ_{2a} chain specific) antibodies (Southern Biotechnology Associates, Inc. Birmingham, AL), were used. Following washing, plates were incubated with the substrate, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; Sigma) and read using an ELISA reader.

Detection of SLE associated manifestations

Leucopenia was determined by mixing heparinized blood with 1% acetic acid at a ratio of 1:10 and the white blood cells (WBC) were immediately counted. Proteinuria was measured in a semi-quantitative manner, using a Combistix kit (Ames–Miles, Slough, UK).

Immunohistology

Kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 6 μm were air dried and fixed in acetone. For the detection of immunoglobulin deposits, sections were incubated with fluoroscein isothiocyanate

(FITC)-conjugated rabbit anti-mouse IgG (γ chain specific, Sigma). Specific staining was visualized using a fluorescence microscope.

Stimulation of cells for cytokine production

Peritoneal macrophages (10^6/ml) pooled from each mouse group were incubated with or without lipopolysaccharide (LPS) (10 μg/ml) in RPMI containing 10% fetal calf serum (FCS) for 24 hr. Supernatants were tested for the production of interleukin (IL)-1. Splenocytes (5×10^6/ml) pooled from each mouse group were incubated with or without concanavalin A (Con A) (2·5 μg/ml) in RPMI containing 10% FCS for 24 hr. Supernatants were tested for the presence of IL-2, IL-4, interferon-γ (INF-γ), IL-10 and tumour necrosis factor-α (TNF-α).

Determination of cytokines

Measurements of cytokine levels were performed by either ELISA or bioassay based on the availability of antibodies for ELISA and the sensitivity of the assay.

IL-1. IL-1 activity was assessed using the LBRM-33(1A5) two-step assay[23] in which 1A5 cells were stimulated in the presence of phytohaemagglutinin (PHA), with the different supernatants at various concentrations to secrete IL-2. Following an overnight incubation, supernatants of 1A5 cells were transferred to the IL-2-dependent cell line CTLL. Stimulation of the CTLL line by IL-2 was then measured by [^{3}H]thymidine uptake.[24]

IL-2. IL-2 was detected using the IL-2-dependent CTLL line.[24]

IL-4. IL-4 activity was assessed using the CT4S cell line.[25] Forty-eight hours following the incubation of the supernatants with the susceptible cells [^{3}H]thymidine was added.

TNF-α. TNF-α levels in the supernatants were determined by the A-9 cell lytic assay using the tetrazolium salt MTT which measures the activity of various dehydrogenase enzymes. This colorimetric assay measures only living cells and is read on a scanning multiwell spectrophotometer (ELISA reader) after 18 hr of incubation of the A-9 cells with the supernatants to be assayed.[26]

IL-10. IL-10 levels in the supernatants were determined by ELISA. Maxisorb plates were coated with 2 μg/ml of monoclonal antibody anti-mouse-IL-10 (JES5-2A5, Pharmingen, San Diego, CA) for 18 hr. Thereafter plates were washed, blocked and the supernatants were added for 24 hr. Following further washing, a biotinylated second antibody anti-mouse-IL-10 (SCC-1, Pharmingen) was added for 60 min incubation. The plates were then washed, and peroxidase–streptavidin (Jackson) was added. Enzyme activity was evaluated using ABTS as substrate.

IFN-γ. IFN-γ was tested by an ELISA assay using a rat anti-mouse IFN-γ monoclonal antibody (R4-6A2 Pharmingen) as a capture antibody in combination with the biotinylated detecting monoclonal antibody XMG1.2 (Pharmingen). The cytokine secretion was determined as described above for IL-10.

Cytokine levels were calculated using standard curves and are expressed as units/ml, except for TNF-α activity that is expressed as ng/ml. The levels of cytokines were determined for supernatants of cells taken from three individual experiments.

Statistical analyses

Significance was determined using either two-tailed unpaired student's *t*-test, χ^2-test with Yates continuity correction or Fisher exact test. A *P*-value of less than 0·05 was considered to be significant. Correction of the *P*-value (Pc) was performed by multiplying the *P*-value with the possible combination pairs from the six different groups tested (i.e. 15).

RESULTS

Autoantibody responses in 16/6 Id injected and treated mice

BALB/c female mice were immunized with 1 μg of the 16/6 Id in CFA. Three weeks later the mice were boosted with the same dose of the human monoclonal autoantibody in aqueous solution. Three weeks following the booster injection, when autoantibody titres in the injected mice were elevated, the mice were divided into groups as follows: Group 1 was not further treated, group 2 was injected twice a week with the oestrogen antagonist tamoxifen (800 μg per mouse s.c.) and Group 3 was injected once a week with the monoclonal anti-oestrogen antibody (50 μg per mouse i.p.). Three other groups served as control groups. The first consisted of mice that were not treated at all, mice of the second group were injected with tamoxifen but were not injected previously with the 16/6 Id and the third group of control mice was treated with the anti-E_2 antibody in the same manner as the experimental mice that were injected with the 16/6 Id for experimental SLE induction. The mice were bled monthly and their sera were tested for the presence of antibody and autoantibody titres. The above experiments were performed three times.

Figure 1 demonstrates representative results of the antibody titres of one experiment determined in the sera of BALB/c mice bled after 2 months of treatment. The figure demonstrates antibody titres specific to the 16/6 Id (immunizing antibody), anti-16/6Id, ssDNA and nuclear extract antigens. As can be seen in the figure, no significant differences could be observed in the antibody levels measured in the sera of the different groups. Thus, the mice produced high antibody titres to the above antigens and neither tamoxifen nor anti-E_2 treatment affected the antibody responses. In fact the antibody levels measured to nuclear extract antigens appeared to be higher in the group that was injected with the 16/6 Id and treated with tamoxifen; however, the differences observed were not significant. No significant antibody activity could be detected in either the non-immunized or tamoxifen treated groups. Low titres of anti-16/6 Id antibodies were observed in sera of the non 16/6 Id immunized and anti-E2 treated mice. However, the latter antibody levels were significantly lower ($P < 0·02$) than those measured in the 16/6 Id immunized mice (Fig. 1).

Effect of treatment on the SLE related clinical manifestations

It was of interest to find out whether treatments with tamoxifen and anti-oestrogen modulated the clinical manifestations characteristics to experimental SLE. To this end, the different mouse groups were tested for the possible presence of leucopenia and proteinuria 5 months after they received the booster injection of the 16/6 Id (3·5 months after their treatment has commenced). Table 1 demonstrates representative results (one out of three experiments) of the white blood cell (WBC)

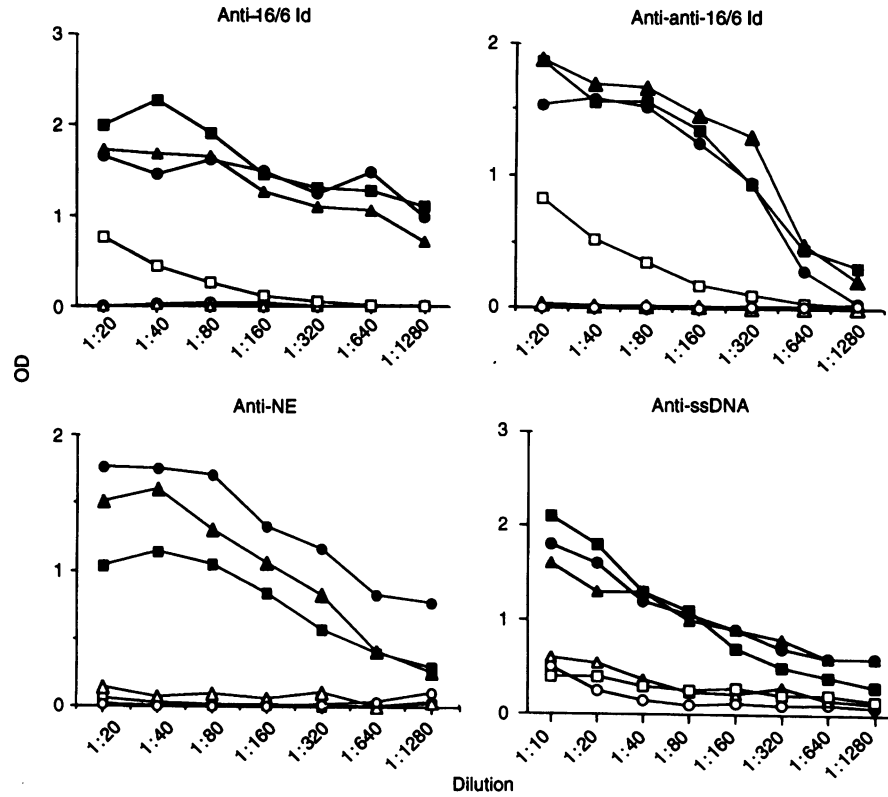

Figure 1. Autoantibody responses in 16/6 Id injected and treated BALB/c mice. Antibody titres to 16/6 Id, rabbit anti-16/6 Id (anti-anti-16/6 Id), ssDNA and nuclear extract (NE) were assessed in sera of individual mice immunized with mAb 16/6 Id (■), and in mAb 16/6 Id immunized mice that were treated with either anti-E2 (▲) or tamoxifen (●). The mice were bled after 2 months of treatment. Control mice were injected with either anti-E2 (□) or tamoxifen only (△), or were not injected at all (○). Results are expressed as mean OD of 10–15 mice tested per group. Standard deviation did not exceed 10% in all assays.

Table 1. Effect of tamoxifen and anti-E2 treatment on the clinical manifestations of 16/6 Id immunized BALB/c mice

Group	Treatment	*Mean WBC ± SD	†Proteinuria mean (g/l ± SE)	‡Immune complex deposits
1	16/6 Id	3490 ± 630	0·23 ± 0·03	12/15 (80%)
2	16/6 Id + anti-E2	5880 ± 300§	0·05 ± 0·01**	3/15 (20%)‡‡
3	16/6 Id + tamoxifen	5040 ± 1000¶	0·03 ± 0·01††	1/10 (10%)§§
4	Anti-E2	6300 ± 1500	0·00	1/9 (11%)
5	Tamoxifen	5800 ± 160	0·03 ± 0·01	0/9 (0%)
6	No treatment	7150 ± 320	0·00	1/12 (8%)

*Number of white blood cells (WBC) per $mm^3 \pm$ S.D.

†Proteinuria, g/l ± SE.

‡Number of mice with kidney deposits per group.

§Significantly different ($pc < 0·008$) from 16/6 Id immunized mice (group 1) but does not differ significantly from anti-E2 injected mice (group 4).

¶Significantly different ($pc < 0·008$) from 16/6 Id immunized mice (group 1) but does not differ significantly from tamoxifen injected mice (group 5).

**Significantly different ($pc < 0·008$) from group 1 but does not differ significantly from group 4.

††Significantly different ($pc < 0·008$) from group 1 but does not differ significantly from group 5.

‡‡Significantly different ($pc < 0·0049$) from group 1 but does not differ significantly from group 4.

§§Significantly different ($pc < 0·0009$) from group 1 but does not differ significantly from group 5.

counts in the different groups of BALB/c mice. It can be seen in the table that mice immunized with the 16/6 Id without further treatment had a mild (but significant) leucopenia. The WBC counts in all the other groups were normal. No significant differences could be observed in the WBC counts of the three control groups and of the groups of mice that were injected with the 16/6 Id and then treated with either tamoxifen or the monoclonal anti-oestrogen antibody. Thus, the latter treatment protocols reversed the low WBC counts observed in mice with experimental SLE to normal levels.

Furthermore, Table 1 shows that mice that were injected with the 16/6 Id had proteinuria ($0{\cdot}23 \pm 0{\cdot}03$ g/l) in comparison to mice in the groups that were injected with the 16/6 Id and treated with anti-E2 ($0{\cdot}05 \pm 0{\cdot}01$ g/l) and with tamoxifen ($0{\cdot}03 \pm 0{\cdot}01$ g/l). The values measured for the latter groups are comparable to those determined for the control groups (non-treated (0·00 g/l), anti-E2 treated ($0{\cdot}03 \pm 0{\cdot}01$g/l) and tamoxifen treated (0·00 g/l) groups). Thus, the treatment of BALB/c mice with either tamoxifen or anti-E2 prevented the development of proteinuria.

Since kidney damage manifested by deposition of immune complexes in the kidneys of the SLE afflicted mice is one of the major characteristics of experimental SLE, all the experimental mice were killed 7 months following the booster injection of the 16/6 Id and their kidneys were evaluated for the presence of immune complex deposits. Table 1 summarizes the results of kidney analyses in all the experimental groups. It can be seen that whereas 80% (12/15) of the 16/6 Id immunized BALB/c mice that were not further treated had multiple immune complex deposits in their kidneys, only 20% (3/15) and 10% (1/10) of the mice treated with anti-E2 and tamoxifen, respectively, were found to have immune complex deposits. These percentages were comparable to those determined in the control groups (8%, 11% and 0% in the non-treated, anti-E2-treated and tamoxifen-treated control groups, respectively; Table 1).

Figure 2 demonstrates representative kidney sections of SLE afflicted BALB/c (Fig. 2a) mice (without treatment) in comparison to kidney sections of mice treated with anti-E2 (Fig. 2b) and with tamoxifen (Fig. 2c) following 16/6 Id immunization. It can be seen that whereas intense immune complex deposits are seen in the kidney sections of the SLE afflicted mice the kidney sections of the treated mice are free of immune complexes.

Isotypes of the anti-DNA antibodies determined in 16/6 Id immunized mice

Since no significant difference was found in total immunoglobulin titres of anti-DNA antibodies in the sera of 16/6 Id immunized treated and untreated mice (Fig. 1), it was of interest to find out whether the beneficial effects of the tamoxifen and anti-E-2 treatment on the clinical manifestation of the 16/6 Id immunized mice, correlate with the isotypes of the autoantibodies produced in the mice. Figure 3 demonstrates that the ratio of IgG2a/IgG_1 of anti-ssDNA antibodies is very high in the group of mice that was 16/6 Id immunized but not treated. In contrast, lower titres of IgG_2a as compared to IgG_1 of anti-ssDNA antibodies were detected in the sera of 16/6 Id immunized mice that were treated with either tamoxifen or the monoclonal anti-E-2 antibody.

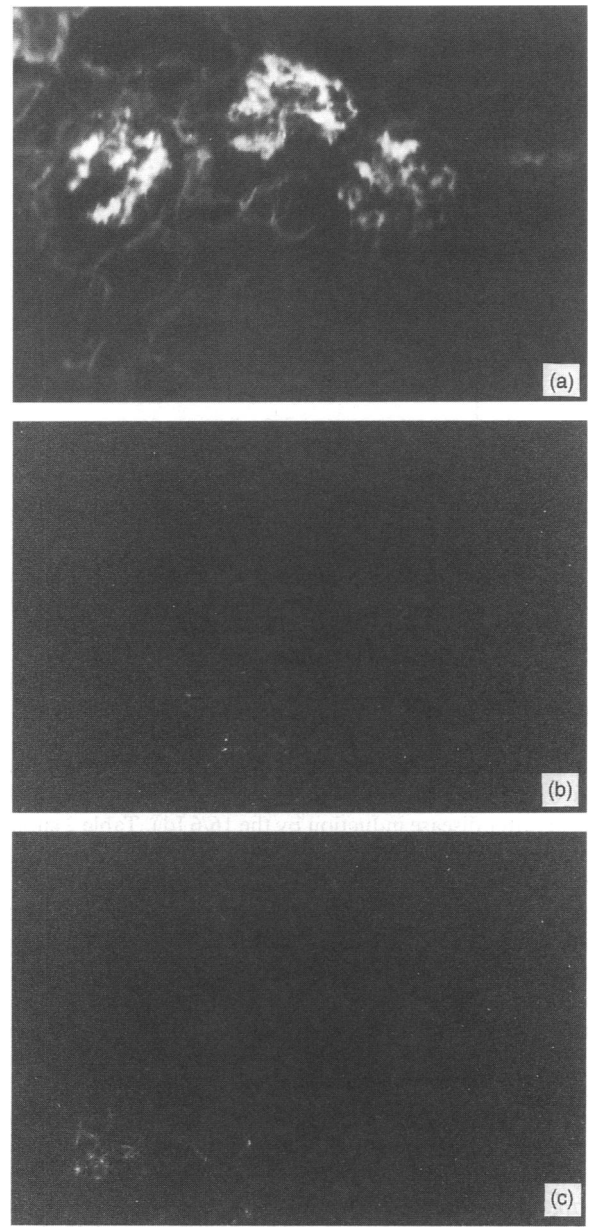

Figure 2. Immunohistology of kidney sections 6 months following the booster injection with the 16/6 Id. BALB/c female mice were immunized with the 16/6 Id and treated 3 weeks after the booster injection with either tamoxifen or anti-E2. Mice were killed 6 months following the booster injection and kidney section were stained with FITC-labelled goat anti-mouse IgG. Immunoglobulin deposits in the kidney section of the 16/6 Id immunized untreated mouse (a), but not in kidneys obtained from the 16/6 Id immunized and anti-E2 (b) or tamoxifen (c) treated mice, are demonstrated. (magnification ×130).

Cytokine profile in treated and non-treated 16/6Id immunized mice

We have previously demonstrated dysregulation in cytokine production of mice with experimental SLE.[17] It has been therefore of interest to find out whether the beneficial effects of tamoxifen or the monoclonal anti-oestrogen antibody (anti-E2) correlate with cytokine modulation in the 16/6 Id immunized mice. For this purpose the cytokine profile was

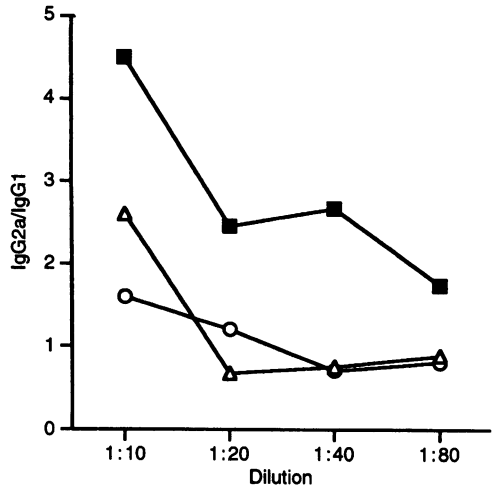

Figure 3. Isotypes of anti-DNA antibodies of 16/6 Id injected mice. Plates were coated with ssDNA, and sera of BALB/c mice immunized with the monoclonal 16/6 Id antibody (■) and of 16/6 Id immunized mice that were treated with either anti-E2 (△) or tamoxifen (○) were added at different dilutions (1:10–1:80). Goat-anti mouse IgG1 or IgG2a coupled to horseradish peroxidase were then added for the detection of antibodies of the above isotypes. Results are expressed as ratio of OD measured for IgG_{2a} and IgG_1 at each dilution.

tested in supernatants obtained from macrophages or spleen cells of mice of the different experimental groups (killed 6 months after disease induction by the 16/6 Id). Table 2 summarizes the cytokine levels measured in the supernatants of LPS-stimulated macrophages or Con A-stimulated spleen cells of the experimental mice in one experiment out of three performed. It can be seen in Table 2 that IL-1 production was significantly higher in the 16/6 Id immunized mice that were not further treated. As seen in the table, treatment with either tamoxifen or anti-E2 reduced the levels of IL-1 secretion to levels comparable to those observed in the control groups. Elevated levels of TNF-α and IL-10 could be detected in the supernatants of splenocytes of 16/6 Id immunized and non-treated BALB/c mice. As in the case of IL-1, both treatment protocols reduced the levels of TNF-α and IL-10 to levels comparable to those of the control groups. It is also seen in the table that reduced levels of IL-2, IFN-γ, and IL-4, were measured in supernatants of splenocytes taken from 16/6 Id immunized BALB/c mice. Results shown in Table 2 indicate that treatment with both tamoxifen and anti-E2 led to secretion of levels of the latter cytokines that are close to those measured in the control mice. It is noteworthy that spontaneous production (without stimulation) of all the above cytokines by macrophages and splenocytes was measured as well. Spontaneous production of only the pro-inflammatory cytokines (i.e. IL-1 and TNF-α) could be detected. Similarly to the results following mitogenic stimulation IL-1 production by macrophages of 16/6 Id immunized mice was significantly higher (12 273 units/ml) than that determined for control mice (225 units/ml). Treatment with either tamoxifen or anti-E2 led to spontaneous production of IL-1 that was lower than that observed for control mice (53 and 128 units/ml, respectively). Similar results were observed when spontaneous TNF-α production was measured by splenocytes of the experimental mice. Thus, 102·4 ng/ml TNF-α was determined in supernatants of splenocytes of 16/6 Id immunized mice as compared to 12·8 ng/ml determined for control mice and 38·4 and 25·6 ng/ml for tamoxifen- and anti-E2-treated mice, respectively. It is noteworthy that similar results were obtained in the two additional experiments performed (standard deviations did not exceed 10%).

DISCUSSION

In this study therapeutic effects have been observed using the oestrogen-antagonist tamoxifen and anti-oestrogen antibody (anti-E2) on the clinical manifestations of the 16/6 Id induced experimental SLE. Furthermore, the improvement in clinical manifestation is associated with cytokine manipulation to normal levels.

Tamoxifen is a synthetic non-steroidal anti-oestrogen compound which binds specifically to the oestrogen receptor in the target organs. The latter has been used to treat women with breast cancer for nearly 20 years and has been proven to have beneficial effects on survival of breast cancer patients without having severe adverse effects.[27,28] We have previously shown that treatment with tamoxifen resulted in significant therapeutic effects on experimental SLE.[13] However, the mechanism by which tamoxifen ameliorated the manifestations of experimental SLE has not been clear. The fact that the anti-oestrogen antibody treatment had similar effects to that of tamoxifen on the 16/6 Id immunized mice (Figs 1 and 2, Tables 1 and 2) suggests that tamoxifen affected the mice by

Table 2. Effect of tamoxifen and anti-E2 on cytokine production of BALB/c mice injected with 16/6 Id

Cytokine activity units/ml	IL-1 Bioassay	TNF-α Bioassay	IL-2 Bioassay	IFN-γ ELISA	IL-4 Bioassay	IL-10 ELISA
Control	2152	25	288	362	147	25
16/6 Id	153 654	153	96	121	37	128
16/6 Id + anti-E2	3532	20	336	274	145	37
16/6 Id + tamoxifen	3271	25	344	283	150	30

IL-1 production by pooled peritoneal macrophages. All other cytokines by pooled splenic cells. Macrophages were stimulated with LPS. Splenocyes were stimulated with Con A. Cytokine levels were calculated using standard curves and are expressed as units/ml except for TNF-α where activity is expressed as ng/ml. The control group consists of mice that were either not treated or treated with either tamoxifen or anti-E2.

its anti-oestrogen capacity. The rationale for the treatment of mice afflicted with experimental SLE with an anti-oestrogen antibody and with tamoxifen is based on our observation that administration of oestrogen to orchiectomized BALB/c male mice prior to their immunization with the 16/6 Id resulted in a very active disease as compared to the mild disease observed in BALB/c males that were immunized with the 16/6 Id.[10] In addition, previous reports described amelioration by testosterone and exacerbation by oestrogen of the SLE like disease of the SLE-prone MRL/lpr and (NZB × NZW)F1 mice.[7,29] Further, a modest beneficial effect of the anti-oestrogen Nafoxidine on the SLE of (NZB × NZW)F1 mice was observed.[30] As for the anti-oestradiol antibody, previous studies have demonstrated that when the latter was given on the day of dioestrus to cycling female rats, it blocked the luteinizing hormone surge and ovulation. In addition, immunoneutralization with anti-E2 of circulating oestradiol, blocked the midcycle synaptic plasticity in cycling rats (F. Naftalin, G. Mor, T.L. Horvath, S. Luquin, A.B. Fajer & L.M. Garcia-Sequva, unpublished observations). Both treatment protocols modulated the clinical manifestations characteristic of experimental SLE, resulting in a significant therapeutic benefit. The 16/6 Id immunized and tamoxifen or anti-oestrogen treated mice had normal counts of WBC and no proteinuria as compared to the significant leucopenia and proteinuria observed in the untreated SLE afflicted mice (Table 1). In addition, both treatments completely prevented the immune complex glomeruloneprhitis that was observed in the 16/6 Id immunized and untreated mice (Fig. 2).

Neither the tamoxifen nor the anti-oestrogen antibody treatments affected the autoantibody responses in 16/6 Id immunized BALB/c mice (Fig. 1), as measured by determination of total IgG autoantibody levels.

Although the anti-DNA antibody titres in the 16/6 Id immunized untreated and treated groups of mice were shown to be similar (Fig. 1), it is noteworthy that the ratio of IgG2a/IgG1 of the anti-DNA antibodies was reduced in the sera of the tamoxifen and anti-oestrogen treated mice (Fig. 3). Indeed, DNA-specific antibodies of the IgG_{2a} isotypes have been suggested to be more pathogenic[31] in agreement with our observation that the treatment with tamoxifen or anti-oestrogen had beneficial effects on the clinical manifestations in the 16/6 Id immunized mice. The mechanisms underlying the pathogenicity of the autoantibodies of the IgG_{2a} isotypes are not known. It might be due to properties such as better capacity to bind complement, to form immune complexes or others. Nevertheless, it is very likely that the shift in anti-DNA isotypes shown in the present study plays a role in the amelioration observed in the clinical manifestations of the treated mice.

The relationship between the immune and neuroendocrine systems has been reported and cytokines were suggested to play an important role in these interactions. The stimulated immune system produces cytokines which may act at various levels of the hypothalamo–pituitary–adrenal axis.[32–35] IL-1 is the most potent cytokine that stimulates hormone secretion. It seems to induce the release of adrenocorticotropic hormone (ACTH) and corticosterone and to participate in the regulation of other hormones such as growth hormone, prolactin and thyroid stimulating hormone (TSH).[32–35] On the other hand, oestradiol has been found to stimulate the production of IL-1 by human peripheral monocytes and maximum release of IL-1 by murine peritoneal macrophages was observed when the highest peak of oestradiol was produced during pregnancy.[36] The other inflammatory cytokines, IL-6 and TNF-α were reported to share with IL-1 most of its biological actions.[32–35,37,38] Further, the T-cell cytokine IL-2 was also demonstrated to act on the hypothalamic–pituitary–adrenal axis *in vitro* and *in vivo* and to regulate the secretion of ACTH, corticosterone, prolactin, luteinizing hormone, growth hormone (GH), TSH, etc.[38–42] In agreement with the above, we have demonstrated in the present study that both treatment protocols, namely tamoxifen and anti-oestrogen antibody, resulted in the modulation of the secretion of all cytokines tested to normal levels (Table 2). Thus, immunocytes of SLE afflicted mice secrete elevated levels of IL-1, IL-10 and TNF-α, and reduced levels of IL-2, IL-4 and INF-γ[21] (see Table 2). Following treatment with either tamoxifen or the monoclonal anti-oestrogen anti-E2 antibody, the levels of all the above cytokines were restored to those determined for immunocytes of healthy mice (Table 2).

The increase in IL-1 and TNF-α (spontaneous or mitogen stimulated) is the most remarkable change occurring in the cytokine profile in experimental SLE. The elevated levels of the latter are compatible with the inflammatory process associated with the disease and reflects the hyperactivation of cells that are responsible for the over-production of IL-1 and TNF-α. These cytokines may be responsible for the dysregulation observed in the production of other cytokines which result in the progression of the pathological manifestations of the disease. It is very likely that the tamoxifen and anti-oestrogen treatment protocols modulate the production of these pro-inflammatory cytokines. As a result, the production of other cytokines is affected. We have recently demonstrated that treatment of mice in which experimental SLE has been induced with the anti-inflammatory drug methotrexate mitigated disease manifestations by down-regulating the levels of the pro-inflammatory cytokines which resulted in the reversal of all other cytokines to normal levels.[43] It thus appears that treatment protocols that lead to a correct cytokine balance may play an important role in the amelioration of the clinical manifestations of experimental SLE.

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